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CAECAL MICROFLORA OF CLETHRIONOMYS GAPPERI

by

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies for acceptance, a thesis entitled Caecal Microflora of Clethrionomys gapperi submitted by Frederick Thomas Theaker in partial fulfilment of the requirements for the degree of Master of Science.

ABSTRACT

The caecum and caecal microflora of *Clethrionomys gapperi* were studied in animals trapped in two habitats, a black spruce-feathermoss consociation and a white poplar stand, for three consecutive seasons. The caecum increased in size and weight from autumn to winter and then decreased the following spring. This was attributed to changes in fiber content in the food.

The most numerous organisms cultured from the caecum were members of the genera *Lactobacillus* and *Streptococcus*. Along with the bacteroides group that were seen in stained smears they constitute the autochthonous flora and their total number increased from autumn to winter to spring. The normal microflora includes the above plus the genus *Staphylococcus*, members of the family Enterobacteriaceae and the yeast *Rhodotorula glutinis*. The literature review and the discussion show that these organisms aid the host by synthesis of vitamins and protein, breakdown of plant cellulose, development of the host's resistance to infection and by warding off infection by pathogenic bacteria and parasitic worms.

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I would like to give special recognition to my wife, Jane, for her encouragement, patience, and assistance in all my endeavours.

If, therefore, anyone wishes to search out the truth of things in serious earnest, he ought not to select one special science; for all the sciences are conjoined with each other and interdependent; he ought rather to think how to increase the natural right of reason, not for the purpose of resolving this or that difficulty of scholastic type, but in order that his understanding may enlighten his will to its proper choice in all the contingencies of life. In a short time he will see with amazement that he has made much more progress than those who are eager about particular ends, and that he has not only obtained all that they desire, but even higher results than fall within his expectations.

--Descartes.

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INTRODUCTION

Bacteria of the rumen have been studied for many years (Hungate, 1966). It is now well established that many of them decompose cellulose to form volatile fatty acids which are absorbed by the rumen and serve as the host's main energy source (Goodall and Kay, 1965; Phillips and Black, 1966; Stevens and Stettler, 1966). Cellulolytic bacteria have also been found in great numbers in the caecum of laboratory rabbits (Hall, 1952). It has also been shown that cellulose is degraded in the caecum of wild rabbits and beaver (Currier *et al.*, 1960), and, in the porcupine the main products of this action are volatile fatty acids (Johnson and McBee, 1967).

Study of laboratory mice has shown their intestinal bacteria to be important in development of intestinal lymphatic tissue and production of gamma globulins (Dubos, 1965 and Luckey, 1965). Many vitamins are synthesized by intestinal bacteria and absorbed by the host (Olcese *et al.*, 1948; Memeesh *et al.*, 1959; Daft *et al.*, 1963; Morgan *et al.*, 1964; Merzbac and Grossowicz, 1965; Klipstein and Samloff, 1966). The host gains the benefit of other synthesized vitamins, along with the bacterial protein, by coprophagy, which is widely practised by rodents and lagomorphs (Southern, 1940; Lechleitner, 1957; Barnes *et al.*, 1963; and Broadbooks, 1965).

The purpose of this study was to investigate the caecal microflora of the red-backed vole, Clethrionomys gapperi. For this purpose animals were captured in white poplar, Populus tremuloides, and black spruce, Picea mariana, habitats for three consecutive seasons; autumn 1965, winter and spring 1966. The caecum was measured and its contents cultured anaerobically. All isolates were presumptively identified using various bacteriological media and stained smears. The knowledge of what bacteria are present in Clethrionomys indicates to what extent the information recorded in the literature review is applicable to Clethrionomys.

LITERATURE REVIEW

Normal Microflora

The autochthonous intestinal flora of most mammals, those microbial species which have achieved a symbiotic status through a long evolutionary association, includes species of Lactobacillus, anaerobic Streptococcus and Bacteroides and, possibly, the fusiform bacteria (Dubos, 1965). Lactobacilli and anaerobic streptococci become established throughout the gastrointestinal tract of laboratory mice immediately after birth. They presumably are derived from the mouth of the mother as she cleans them (Dubos et al., 1967). The bacteroides group does not become established until after the second week of life and then only in the caecum and large intestine, but persist there in very large numbers (Schaedler et al., 1965). The development of the intestinal flora of calves, lambs, piglets, puppies, kittens, rabbits, guinea-pigs, rats and chickens was investigated by Smith (1965b). He also found that the bacteroides group was slower in colonizing the alimentary tract than the other autochthonous bacteria and that they were restricted to the large intestine and were the principal inhabitants of most of the animals studied. The first bacteria to colonize the alimentary tract of most of these animals were Escherichia coli, Clostridium

perfringens and streptococci. These were followed by lactobacilli, which became the commonest inhabitant in the stomach and small intestine of animals other than the puppy, kitten and rabbit. E. coli and Cl. perfringens presumably were derived from early contact with adult feces and were able to multiply because of the high pH of the stomach in young animals. The subsequent lower pH in the stomach with advancing age may be less disadvantageous to the lactobacilli and may account for the change in microflora (Smith, 1965b). Suckling rabbits were unique in that their stomach and small intestine were often almost completely sterile. This has been attributed to an antimicrobial substance produced in the stomach by enzymatic action on the chloroform-soluble fraction of rabbit milk (Smith, 1966). Staphylococcus aureus, common in puppies, was probably derived from the breast of the dam. Yeasts were uncommon in animals on a diet of milk. Guinea-pigs often began eating with their parents during their first day and yeast was a major component of their microflora.

Bacteroides and Lactobacillus were the predominant genera found in the intestine of laboratory-reared Syrian hamsters, Mesocricetus auratus (Hagen et al., 1965). Species of the genera Streptococcus, Bacillus, and species of the family Enterobacteriaceae and also Staphylococcus,

in descending order of frequency, were also isolated. Kunicki-Goldfinger and Kunicka-Goldfinger (1962a) studied the intestinal flora of the European common shrew Sorex araneus araneus and the European red-backed vole Clethrionomys glareolus glareolus in Poland's national park at Bialowieza during four successive seasons. They reported that streptococci and coccoidal 'K-forms' (originally described by Rettger and Horton, 1914) were a prominent part of the normal microflora of Clethrionomys whereas lactobacilli were very sparse. Species of Enterobacteriaceae, Micrococcus and Bacillus were also prominent. Cellulolytic species of Clostridium were present in the caecum.

Both Schaedler et al. (1965) and Smith (1965b) found that, in the young animals they studied, the numbers of E. coli reached a peak and then diminished. This was also reported by Mushin and Dubos (1965) who showed that young laboratory mice were highly susceptible to intestinal infection by an enteropathogenic strain of E. coli. The period of highest susceptibility extended from birth to about two weeks of age. Adult mice failed to become infected and all mice, irrespective of their age of infection, had a striking decrease in their E. coli population between 24 and 28 days of age. This is suggestive

evidence that resistance with age is related to the development of an intestinal microflora which is antagonistic to E. coli. Thus E. coli, which is the most commonly known bacterium, may be part of the normal flora but is not considered a part of the autochthonous flora of mammals.

Contrary to reports from earlier studies (Donaldson, 1964) the stomach and small intestine are not sterile. Smith (1965a) studied adults of 20 species of animals including domestic mammals and birds, monkeys, Macaca irus; tortoises, Testudo sp.; frogs, Rana temporaria; cockroaches, Periplaneta americana; and fishes, Ritulus ritulus, and Gadus merlangus. In homotherms he found the normal bacteria in all parts of the gastrointestinal tract; E. coli, Cl. welchii, streptococci, lactobacilli, bacteroides and yeasts. Counts were higher in the cardiac portion of the stomach, especially in coprophagous animals, than in the pyloric portion where a low pH reduces the numbers. Bacteria occur in increasing numbers from the duodenum, jejunum and ileum to the caecum and colon where their numbers are highest. Low numbers in the small intestine have been attributed to clearance by peristalsis (Dixon, 1960). The contents of the caecum and colon are relatively stationary, thus allowing the bacterial population to

increase. Lev et al. (1966) showed that the Lactobacillus population in the caecum of laboratory rats was stable even when coprophagy was prevented.

Rosebury (1962) and Donaldson (1964) reported a similar distribution of bacteria in the intestine of man. The microflora is predominantly obligate anaerobic and dominated by species of Bacteroides and Lactobacillus. Buchan and Gould (1967) include clostridia in this group.

The study of germ-free animals has stimulated interest in the caecum and its microflora. Wostmann and Bruckner-Kardoss (1959) showed that germ-free laboratory rats (also guinea-pigs and mice), compared to "conventional" animals of the same age, exhibited an increase in caecal weight which started during the second week of life. Caeca of adult animals showed a five- to seven-fold increase in size when compared to caeca of "conventional" animals. Gordon and Wostmann (1959) observed that the establishment of a normal intestinal flora in germ-free rats caused the caecum to recede to conventional proportions within a week. A diet with more fiber has also been found to reduce the size of the caecum of germ-free animals from 30% to 15% of the body weight (Luckey, 1965).

Normal Microflora and Host Nutrition

The intestinal bacteria aid ruminant and non-ruminant herbivores by the breakdown of cellulose and starch. Food eaten by ruminants is digested in three stages: (1) it is fermented by bacteria in the rumen where two-thirds of the organic matter is degraded and absorbed (Goodall and Kay, 1965); (2) the residue, saliva and microorganisms pass into the abomasum and small intestine to be digested by acids and enzymes; and (3) the waste products and remaining residue are acted upon by bacteria in the large intestine.

Very little carbohydrate is absorbed as such by ruminants since the microflora of the rumen attack these materials. The volatile fatty acids, acetic, propionic and butyric, are considered to be the main products of rumen fermentation. They are the main energy source in ruminant metabolism (Hungate et al., 1961) and are absorbed directly by the epithelium of the rumen (Stevens and Stettler, 1966; Phillips and Black, 1966).

Food eaten by non-ruminant herbivores is digested in two stages of a recurrent cycle. After digestion and absorption in the stomach and small intestine, food residue is moved to the caecum and large intestine. Here bacterial enzymes break down cellulose and starch (Eden, 1940; Baker et al., 1950; Hall, 1952; Currier et al., 1960). The contents

of the caecum are reingested as fecal pellets thus starting another cycle (Morot, 1882; Osborne and Mendel, 1911; Lechleitner, 1957).

Coprophagy in rabbits was first reported by Morot (1882) and later confirmed by Madson (1939) and Taylor (1939). Morot observed that rabbits produce two sorts of feces and presumed that the soft mucoid feces were consumed for the purpose of cellulose digestion. This idea was substantiated by Hall (1952) who isolated several cellulolytic cocci in significant numbers from the caecal contents of rabbits. Her work was supported by Currier et al. (1960) who showed that cellulose implanted in the caeca of rabbits and beaver and in artificial caeca was reduced in weight 32% to 33%.

Osborne and Mendel (1911) were the first to use the growth of rats as the criterion of the value of a ration. They reported that, on a poor diet, growth improved when laboratory rats were allowed to supplement their diet with their feces. Eden (1940) concluded that coprophagy increased the digestion of fiber in foods: coprophagy in laboratory rabbits varied from 54% to 82% of egested feces. Baker et al. (1950) studied the breakdown of starch in laboratory mice, hamsters, guinea-pigs, rabbits and domestic pigs. Insoluble starch granules reached the caecum and were attacked by bacteria, mainly Clostridium butyricum. Animals fed soluble starches in a vitamin B-free diet were unable to refect.

Barnes et al. (1957) showed that rats ate 50% to 65% of their feces and Thacker and Brandt (1955) showed that any portion of food may pass through the digestive tract up to nine times. Prevention of coprophagy in rats depressed the growth rate 15% to 25% (Barnes et al., 1963).

Coprophagy has been observed many times in wild hares and rabbits (Southern, 1940; Watson, 1954; Hamilton, 1955; Kirkpatrick, 1956; Lechleitner, 1957). Pikas, Ochotona hyperborea, have been seen eating their own feces (Hagen, 1960). Broadbooks (1965) was surprised to find dozens of dried scats of yellow-bellied marmots, Marmota flaviventris, in many hay piles of the pika, O. princeps. He also records that Taylor and Shaw (1927) found coyote, Canis latrans, as well as marmot scats in hay piles of pika on Mt. Ranier. Coprophagy has also been observed in juvenile and adult shrews, Sorex araneus after eversion of their anus (Crowcroft, 1952).

The ability of intestinal organisms to manufacture niacin, thiamine, riboflavin, pyridoxine, vitamin B_{12}, folic acid, pantothenic acid, biotin and vitamin K is well established (Kon and Porter, 1954; Mickelsen, 1956, 1962; Gustafsson et al., 1962). There is good evidence that folic acid, biotin, vitamins B_{12} and K are absorbed by the large intestine, and some evidence for thiamine and riboflavin being absorbed here also (Olcese et al., 1948; Memeesh et al.,

1959; Morgan et al., 1964; Merzbac and Grossowicz, 1965; Klipstein and Samloff, 1966). However, it is likely that non-ruminants must derive the benefit of these vitamins largely as a result of coprophagy (Barnes et al., 1963; Donaldson, 1964; Dubos, 1965).

Valdivieso and Schwabe (1966) showed that medium-chained lipids are absorbed by the caecum of the laboratory rat. They worked with octanoic acid and glyceryl trioctanoate. Volatile fatty acids are known to be absorbed here also (Barry and Smyth, 1960; Barry et al., 1966). Johnson and McBee (1967) showed that acetic, propionic and butyric acids are the chief products of caecal fermentation of the porcupine Erethizon dorsatum. Most of these are absorbed, with 88% of the absorption from the caecum and 12% from the large intestine. The fermentation resembled that of a ruminant eating a high roughage diet and contributed 16% to 33% of the animal's energy requirement. Porcupines were reported to be non-coprophagic.

Protein utilization is affected by coprophagy. Stillings and Hackler (1966) showed that prevention of coprophagy decreased the biological value and nitrogen absorption of a 19% protein diet and increased urinary nitrogen excretion and metabolic fecal nitrogen. Levenson et al. (1959) showed that in laboratory rats urea is freely diffusable from the

blood into the lumen of the alimentary tract where it is converted by the enzyme urease into ammonia. There is possibly some urease in the intestinal mucosa but the greatest amount is bacterial in origin (Einheber and Carter, 1966). Walser and Bodenlos (1959) by means of tracer studies, found that at least 25% of the urea in the body undergoes hydrolysis in the intestine. A practical importance of this is the resultant rise in pH in the intestine. The ammonia also aids bacterial growth and, in ruminants, bacteria are a major source of proteinaceous nitrogen for the host. Coprophagy makes it possible for non-ruminant herbivores to use bacteria as a source of nitrogen.

Normal Microflora and Resistance to Infection

A classification of defense mechanisms has been suggested by Luckey (1965) based on consideration of morphological, physiological and chemical similarities and differences between germ-free and what he calls classic animals. Those defense mechanisms in which germ-free and classic animals have similar capabilities are called "constitutive". Included in this category are skin, nasal hair, saliva, mucosal epithelium, thymus, bursa Fabricii, complement and properidin to name only a few. The second category of

defense mechanisms is definitely underdeveloped in the germ-free state. Therefore microorganisms are directly or indirectly necessary for their development and they are called "adaptive" defense mechanisms. They are induced by the addition of specific bacteria to the germ-free animal and induced naturally in new-born animals. Included in this category are formation of the caecal apical patch, development of lymph nodes, rhythmic caecal contractions, antibody formation, specific gamma globulin formation and development of plasma cells.

The third category of defense mechanisms has been called "extracorporal". This is the indigenous microflora. Members of the genera Lactobacillus and Streptococcus produce large volumes of lactic, acetic, propionic and butyric acid (Skerman, 1959). These inhibit the growth of many bacterial species by their toxic effect, as well as by lowering the pH (Bergeim et al., 1941; Tramer, 1966). Other bacterial species cannot increase to lethal levels because of strong competition for food and living space on the intestinal mucosa by the indigenous flora (Luckey, 1965; Ketyi, 1964). A few of the competitive interactions are listed by Donaldson (1964).

Bacteria also affect the establishment of intestinal parasites (Stefanski, 1965). Newton et al. (1959) were able

to infect germ-free rats with Nippostrongilus muris and germ-free mice with Nematospiroides dubwin but were unable to infect "conventional" animals. On the other hand Entamoeba histolytica could not establish itself in germ-free animals (Phillips and Wolfe, 1959) but no bacterial pattern has been found to be associated with the presence of this parasite (Antia et al., 1965). Another example is that Lamblia intestinalis occurs in large numbers only when Escherichia coli, normally only present in the large intestine, is present in the small intestine (Liebmann, 1953).

Thus the normal intestinal microflora of an animal has a basic composition composed of species of Bacteroides, Lactobacillus and Streptococcus all of which are anaerobic. They are found in greatest numbers in the rumen and/or caecum and large intestine. They aid the host by decomposing cellulose and hard starch granules. At the same time they synthesize vitamins and protein which the host can use. Their presence stimulates the host's defense mechanisms as well as inhibiting the establishment of pathogenic bacteria and intestinal parasites.

Cellulose

Cellulose is the most abundant organic compound in the world, comprising 50% or more of all the carbon in vegetation (White et al., 1959). Complete hydrolysis yields glucose

almost quantitatively whereas partial hydrolysis yields cellobiose, cellotriose and other polymers. Cellulose is present in plant cell walls in the form of elementary fibrils, with a diameter of 35 Å each containing approximately 40 cellulose chains that are held together by hydrogen bonds. The elementary fibrils are aggregated into larger microfibrils that vary in width from 80 Å to 200 Å and half as thick (Timell, 1964) and may reach lengths of 30,000 Å to 40,000 Å (Albersheim, 1965). All cell walls also contain non-cellulose polysaccharides, particularly mannan, xylan, galactan, araban and pectic compounds. These substances are alpha- or beta- 1,4 linked (Preston, 1965).

Early work on the means of degrading cellulose was related to its decomposition in the soil and the means of attack by plant pathogens. The first characteristic of all cellulolytic fungi is the presence of an enzyme C_1. It acts on crystalline cellulose, possibly by breaking the hydrogen bonds and thus releasing the cellulose molecules. This would allow swelling of the molecules by hydration and make them available for action by enzyme C_x. This enzyme is really a complex of enzymes hydrolyzing the beta- 1,4 glucosidic bonds in the cellulose molecule and bringing about random splitting of the polysaccharide to form cellobiose (Mendels and Reese, 1965). The enzymes C_1 and C_x are extracellular whereas the enzyme cellobiase is intracellular, and

cellobiose enters the cells as readily as glucose (Siu, 1951). All cellulolytic organisms must have the enzymes C_1, C_x and cellobiase whereas non-cellulolytic organisms may have the enzyme cellobiase.

STUDY AREAS

Two habitats were selected as study areas. One was an isolated 3-acre stand of white poplar, Populus tremuloides, about two miles west of Ellerslie, Alberta. The other was a stand of black spruce, Picea mariana, one mile west and four miles north of Spruce Grove, Alberta. It was about one acre in area and at the southern end of an extensive forest.

The poplar stand was dominated by white poplar with some black poplar P. balsamifera on the outer third of the stand. The center was dominated by tall white spruce, Picea glauca. Characteristic species of this consociation include Cornus canadensis, Shepherdia canadensis, Merianthemum canadensis, Rosa woodsii, R. acicularis, Rubus idaeus, Salix spp., Pyrola asarifolia, and Fragaria glauca.

The black spruce stand was mainly a black spruce-feathermoss, P. mariana-Hyloconium splendens, consociation which was accompanied by white spruce, white poplar and willow, Salix spp. The forest floor was covered with feathermoss and Pleurozium schreberi. Characteristic species were Cladonia spp., Peltigera aphthosa, Ledum groenlandicum, Vaccinium vitisidaea var. minus, Cornus canadensis, Rosa spp., Ribes spp., Equisetum spp., Petasites palmatus, Linnaea borealis var. americana, Mitella nuda, Rubus pubescens and Carex spp. (Moss, 1955). Larch, Larix laricina, and birch, Betula spp., were also present in some areas.

METHODS

Animals were captured alive in both habitats using Sherman traps. These were set during September and October 1965, March 1966 and May 1966. March represented the winter season with full snow cover. May represented spring and the traps were not set until the new grass was showing. In winter the traps were wrapped with terylene fiber and covered with a plastic bag which was held in place with elastic bands. Terylene fiber was also placed inside the traps for nesting material. These precautions were taken in order to decrease the number of deaths in the traps resulting from heat loss aggravated by low temperature, inactivity and the presence of moisture. All traps were left out approximately eight hours.

The animals were carried to the laboratory in the traps and held there at room temperature without food or water. The time from pick-up until death varied from one and one-half to twelve hours. Animals were killed with ether and only freshly killed animals were used to avoid changes in the bacterial population which might occur after the death of the host.

All animals were weighed and measurements were taken of total length, length of tail and left hind foot. Each animal was then placed in a porcelain tray and its fur soaked with alcohol. After waiting several minutes for the

disinfectant to act the abdominal cavity was opened aseptically with sterile forceps and scissors. The caecum was lifted by its distal end with sterile forceps and the coils stretched out by cutting the mesentery with scissors. It was then tied off with sterile thread at its junction with the intestine and the length of the caecum was measured from this point.

The weight of the caecal contents was determined by weighing the caecum before and after emptying its contents into a sterile ground-glass tissue grinder which had a stream of oxygen-free carbon dioxide gas flowing into it (Skerman, 1959, p. 203). A ten percent weight-volume dilution was then made with a sterile, anaerobic salt solution (see Appendix A) and the contents ground until totally suspended. Hungate (1950) reported that many bacteria adhere to particles and grinding releases them so that a more accurate estimate can be made of the number of bacteria present. Three slides were smeared, each with 0.1 ml sample of the 10^{-1} dilution and later Gram stained and examined. The initial 10^{-1} dilution was then used to make serial ten-fold dilutions up to 10^{-8}, or as needed.

From three consecutive dilutions 0.1 ml quantities were used to inoculate, in triplicate, petri plates of the following media: MacConkey Agar (MA), Sheep Blood Agar (BA), Rogosa Agar (RA), and Phytone Yeast Extract (PYE). The

inoculum was spread on each plate by means of large glass spreaders while the plate was rotated on a Petri Dish Turntable. Rogosa Agar plates and half of the Sheep Blood Agar plates were incubated anaerobically in Brewer's jars under an atmosphere of hydrogen gas. All plates were incubated at 37°C except the PYE which were incubated at room temperature. The aerobic plates were read after 18 hours and the anaerobic plates after 36 hours. Colony counts were recorded from plates with 30-300 colonies.

Triplicate test tubes of Anaerobic Cellobiose Agar (ACA) were inoculated from three consecutive dilutions. The test tubes of melted ACA were held in a water bath at 45°C until used. During inoculation a stream of oxygen-free CO_2 gas (hereafter referred to as CO_2) was directed into the tubes to exclude oxygen. After inoculation each tube was filled with CO_2 gas, stoppered and rapidly rotated by hand under running cold water until the agar formed a solid uniform layer around the inside of the test tube. The test tubes were incubated at 37°C and read after five days, using an Olympus stereoscopic microscope at 20X. Colony counts were recorded for tubes having 30-300 colonies. The above method was devised by Hungate (1950).

The anaerobic test tubes contained Bryant's medium (see Appendix A). The medium was prepared in one litre quantities titrated to pH 6.8 and autoclaved. While it

was cooling sterile cysteine and $NaHCO_3$ were added aseptically while CO_2 gas was bubbled through the medium. Five ml quantities of the complete medium were siphoned into 16 x 150 mm test tubes. While being filled each test tube was treated with CO_2 gas via a bent 6 inch, No. 16 hypodermic needle. The arrangement of the apparatus for maintaining anaerobiosis is shown in Fig. 1. The presence of oxygen caused the resazurin to turn pink. All test tubes with pink agar were discarded.

Bacteria differ in morphology but differ even more in their nutritional requirements. Which bacteria will grow in vitro from a mixed inoculum depends upon which organism's natural environment is reproduced. Thus the use of various Eh, pH, salts and nutrients with varied concentrations, determines which bacteria can grow. Because of these factors, as well as the use of inhibitors, the media are selective for different bacteria. Each can also be made a differential medium by the addition of different indicators. Selective media are a necessity. At high dilutions the less numerous genera will not be detected because of the dilution. At low dilutions the less numerous genera would not be detected because of overgrowth by the more numerous genera. For this reason I used the listed selective media as a means of enumerating the different kinds of bacteria present in my samples. In theory each colony represents one bacterium in the sample.

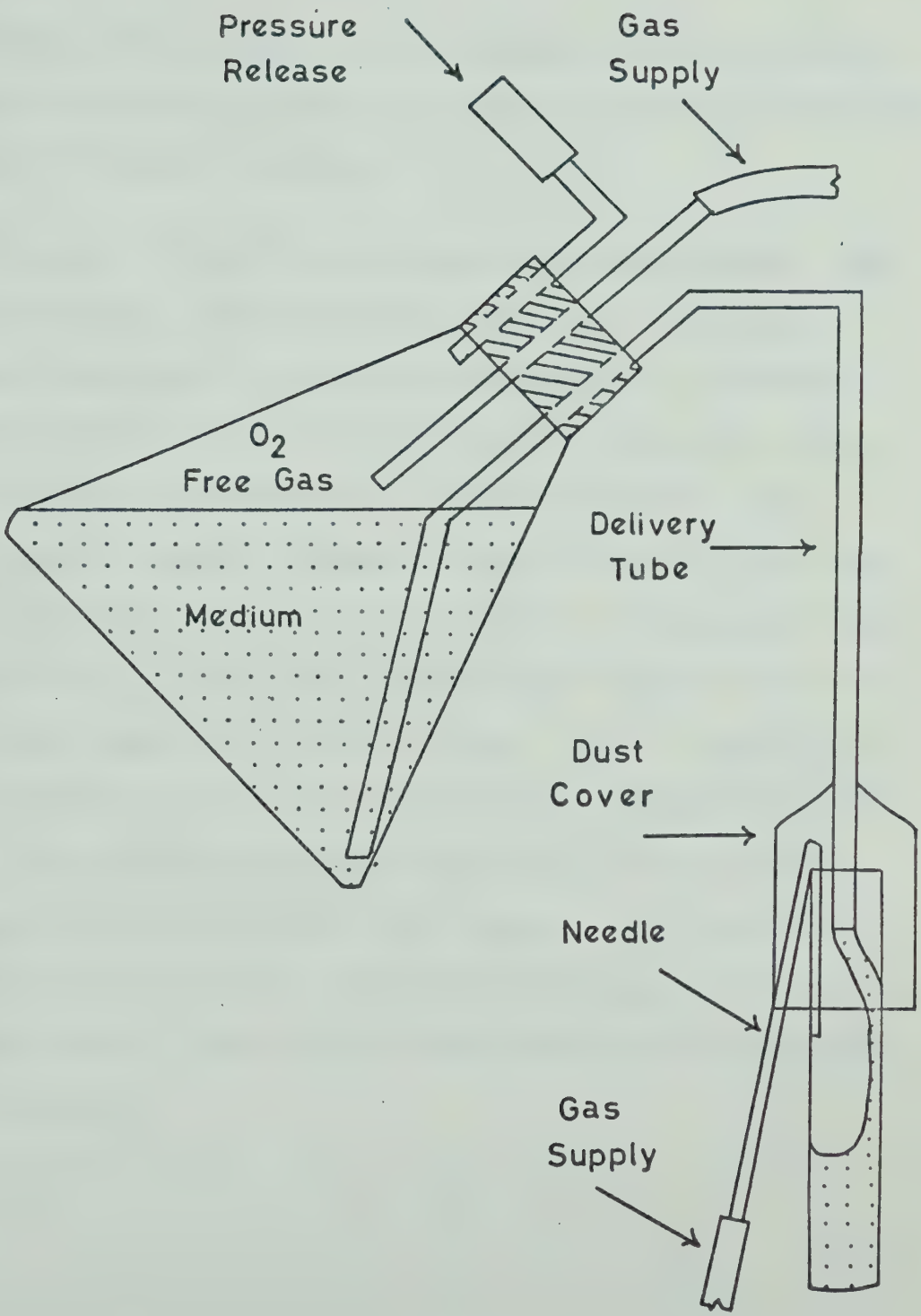


Fig. 1. Apparatus arrangement for maintaining anaerobiosis while preparing a stock of anaerobic roll-tubes.

The average colony count of the replica plates is multiplied by the dilution factor to give the number of bacteria per gram of caecal contents.

Colonies, of each type observed on each medium, were smeared on glass slides and Gram stained. Colonies of each type observed in ACA were transferred to Anaerobic Cellobiose Broth (ACB) with CO_2 directed into the tubes while they were unstoppered. Colonies from ACA were picked using a sterile Pasteur pipette with its tip bent at 90° (see Fig. 2) using mouth suction. When growth was visible in ACB, it was used to inoculate Petri plates of Sheep Blood Agar (BA) and Rogosa Agar (RA). One-half of the BA plates plus the RA plates were incubated anaerobically. Cellulose Agar roll-tubes were also inoculated and inspected each week for five weeks. Representative colonies from each plate and tube were smeared and stained with Gram stain. This information provided a presumptive identification.

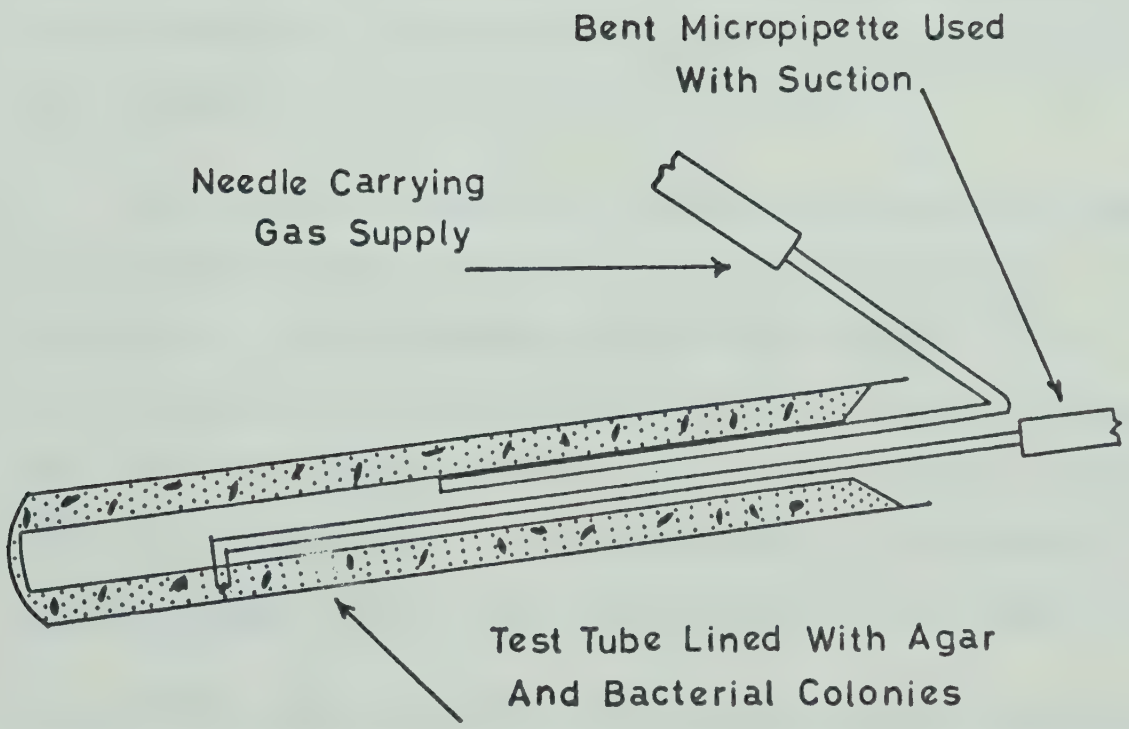


Fig. 2. Selection of a colony anaerobically.

RESULTS

Body Measurements

The average body and caecal measurements of Clethrionomys gapperi are presented in Table 1 and the levels of significant difference, as determined by Student's t-test (Snedecor, p. 90 and 47, 1957) are shown between the averages. The summary of statistics for comparison of groups of different size is presented in Appendix B.

The absolute weights of the body and caecum are not greatly different between the two habitats but are, generally, between seasons within each habitat. This is also seen in the ratio body weight/caecal weight except for the great difference between habitats in the autumn. This is a reflection of the difference in body weight of Clethrionomys between the two habitats at this season.

In the spruce habitat the absolute caecal length changed significantly with each season. This change wasn't evident in the poplar habitat, however no values are available for the autumn. The absolute body length (nose to base of tail) increased significantly from winter to spring in both habitats. The ratio of body length/caecal length differed greatly between habitats in the winter and between seasons only in the spruce habitat.

Table 1. Averages of body and caecal measurements of Clethrionomys gapperi and their ratio with the level of significant difference, by Student's t-test, shown between the averages and sample size in brackets.

Measurement	Habitat	SEASON					
		Autumn		Winter		Spring	
Body Weight (gm)	Poplar	(6)		(15)		(5)	
		16.9	P.01	15.0	P.001	20.65	
		P.025		P.1		P.1	
	Spruce	13.15	P.5	13.9	P.001	18.39	
		(6)		(14)		(5)	
Caecal Weight (gm)	Poplar	(4)		(14)		(3)	
		0.51	P.05	0.94	P.1	0.53	
		P.2		--		P.2	
	Spruce	0.68	P.2	0.94	P.01	0.37	
		(6)		(14)		(5)	
Body Weight / Caecal Weight	Poplar	(4)		(14)		(3)	
		33.2	P.05	19.6	P.025	41.1	
		P.01		P.5+		P.4	
	Spruce	20.0	P.5+	19.9	P.005	58.5	
		(6)		(14)		(5)	

Table 1 (Cont'd.)

Measurement	Habitat	SEASON				
		Autumn		Winter		Spring
Body Length (mm)	Poplar	(4)		(14)		(3)
		86.3	P.4	88.4	P.001	104.
		P.025		P.4		P.5+
	Spruce	91.8	P.5+	90.7	P.005	102.
		(6)		(14)		(5)
Caecal Length (mm)	Poplar	-		(13)		(3)
		-		80.1	P.5	83.3
				P.025		P.5
	Spruce	75.8	P.01	89.1	P.025	80.0
		(6)		(14)		(5)
Body Length / Caecal Length	Poplar	-		(13)		(3)
				1.15	P.2	1.28
				P.01		P.5
	Spruce	1.14	P.01	1.00	P.001	1.32
		(6)		(14)		(5)

Microbiology

Averages of bacterial colony counts on different media are shown in Table 2. These organisms represent the normal microflora of Clethrionomys as determined by the media employed and the classification of Breed et al. (1957).

MacConkey medium is designed to support the growth of intestinal, aerobic Gram-negative rods. Only slow lactose-fermenting rods were isolated and identified as members of the genera Aerobacter and Citrobacter. It is noteworthy that Escherichia coli was never present. These organisms are all members of the family Enterobacteriaceae and they were the least abundant of the bacteria isolated. They were more numerous in animals from the spruce habitat than from the poplar habitat and more numerous in the spring than in other seasons.

Only one species of yeast, Rhodotorula glutinis (Lodder and Kreger van Rij, 1952) was isolated from the caecum when cultured on Phytone Yeast Extract. Its nutritional characteristics are assimilation but not fermentation of glucose, galactose, sucrose and maltose but not lactose and utilization of nitrate as a nitrogen source. The cells were oval, produced pink colonies at room temperature and did not grow at 37°C. This yeast

Table 2. Averages of bacteria per gram of caecal contents by culturing directly from the caecum of Clethrionomys gapperi on selective and differential media.

Medium	Habitat	SEASON		
		Autumn	Winter	Spring
MacConkey Agar	Poplar	-	$10^1 - 10^2$	10^2
	Spruce	-	2.46×10^3	59.8×10^4
Phytone Yeast Extract	Poplar	-	$10^5 - 10^6$	$10^2 - 10^3$
	Spruce	-	33.7×10^3	41.2×10^3
Blood Agar	Poplar	-	77.1×10^6	0.34×10^6
	Spruce	-	1.38×10^6	9.01×10^6
Anaerobic Blood Agar	Poplar	-	713.0×10^6	116.0×10^6
	Spruce	-	2.97×10^6	5.55×10^6
Anaerobic Rogosa Agar	Poplar	-	68.1×10^7	74.7×10^7
	Spruce	-	515.0×10^7	0.38×10^7
Anaerobic Cellobiose Agar	Poplar	34.4×10^8	16.1×10^8	28.3×10^8
	Spruce	43.7×10^8	43.6×10^8	11.8×10^8

appeared in a random manner in animals from each habitat and season. Its numbers varied from fewer than 100 to 260,000 per gram of caecal contents. The average number isolated was fairly constant from animals of the spruce habitat and consistently higher than those of the poplar habitat.

Sheep Blood Agar is used to grow fastidious micro-organisms. Gram positive cocci, species of the genera Streptococcus and Staphylococcus, were the predominant organisms isolated on it. Staphylococci were differentiated from micrococci by stab inoculation of deep tubes of glucose agar. Staphylococci utilize glucose both fermentatively and oxidatively (Subcommittee, 1965). Most isolates were coagulase-negative and so classified as Staphylococcus epidermidis. Staphylococcus aureus was isolated from a few animals but only in small numbers. Gram positive non-sporing rods (lactobacilli) and Gram positive diplococci were also isolated from a few animals on this medium. Bacterial growth on BA was most abundant from the caecum of animals from the poplar habitat during winter and from the spruce habitat during spring. There was a great change in numbers of these bacteria between seasons. From winter to spring there was almost a 200-fold decrease in bacterial number in animals from the poplar habitat. During the same period there was a 9-fold increase in bacterial number in animals from the spruce habitat.

Sheep Blood Agar, incubated anaerobically, supported the growth of Gram positive cocci (species of Streptococcus) and Gram positive non-sporing rods (species of Lactobacillus). Gram negative non-sporing anaerobic rods (members of the bacteroides group) were seen in smears but only from mixed colonies. The results do not permit a generalization concerning the incidence of the different bacterial genera by either season or habitat. Some animals yielded either lactobacilli or streptococci while some yielded both. During the winter anaerobic growth on BA was more abundant from animals from the poplar habitat.. In spring their numbers were reduced but were still greater than those from animals from the spruce habitat.

When the anaerobic and aerobic growth on BA was compared it was evident that anaerobic bacteria were more numerous. A comparison of the growth from animals of the poplar habitat showed a 10-fold difference during the winter which increased to a 200-fold difference in the spring. An exception to this was seen in the spruce habitat in the spring when aerobic growth was more abundant.

Rogosa Agar, incubated anaerobically, was selective for species of Lactobacillus. During winter lactobacilli were more numerous in animals from the spruce habitat. In spring they were more numerous in animals from the poplar habitat. Gram stained smears showed that several species

were present, based on length and shape of cells and colony type. Cells with V- or Y-shaped branching, which is indicative of L. bifidus, were never observed.

Anaerobic Cellobiose Agar supported the growth of anaerobic and facultative anaerobic bacteria. I assumed from statements in the literature that the caecum was an anaerobic environment (Hungate, 1950; Hall, 1952; Zubrychi and Spaulding, 1962) and only digestion-resistant substances such as cellulose and starch granules reached it (Currier et al., 1960; Mickelsen, 1962). Monosaccharides and common disaccharides are believed to be completely absorbed by the small intestine (White et al., 1959; Kohn et al., 1965; Crane, 1966). Thus ACA should support the growth of most of the bacteria living in the caecum. The highest counts were obtained on this medium and the count of 10^8 is comparable with other reports of total anaerobes (Dubos et al., 1965; Hagen et al., 1965).

The bacterial colonies isolated from ACA were transferred to ACB and then to other media including Cellulose Agar for identification. The autumn isolates on ACA were held for many weeks in ACB before being transferred to the other media. Since many bacteria were short-lived in ACB, or never grew, the percentage of different genera isolated must be interpreted with caution.

The percentage of different bacterial genera isolated on ACA is shown in Table 3. In the poplar habitat Gram positive non-sporing anaerobic rods (lactobacilli) were least numerous during autumn while Gram positive anaerobic cocci (streptococci) were intermediate and enterobacteria were most numerous. In winter lactobacilli and streptococci were about equal in number and enterobacteria were never isolated. During spring the isolates were all lactobacilli.

In the spruce habitat, lactobacilli and enterobacteria were equally low in number during autumn while streptococci were prominent. During winter the lactobacilli increased in number whereas the enterobacteria decreased in number. The streptococci also decreased in number but were still the most abundant. In spring lactobacilli were twice as numerous as streptococci while enterobacteria were then present in very low numbers.

Cellulose Agar

Although growth occurred in many tubes, a clearing about a colony, indicative of cellulose degradation, was never observed. A pure culture of Ruminococcus albus, a known cellulolytic organism, was supplied by Dr. M.P. Bryant of the University of Illinois. Although this organism grew in the Cellulose Agar, I did not observe clearing about its colonies. Thus it is likely that the test medium was faulty.

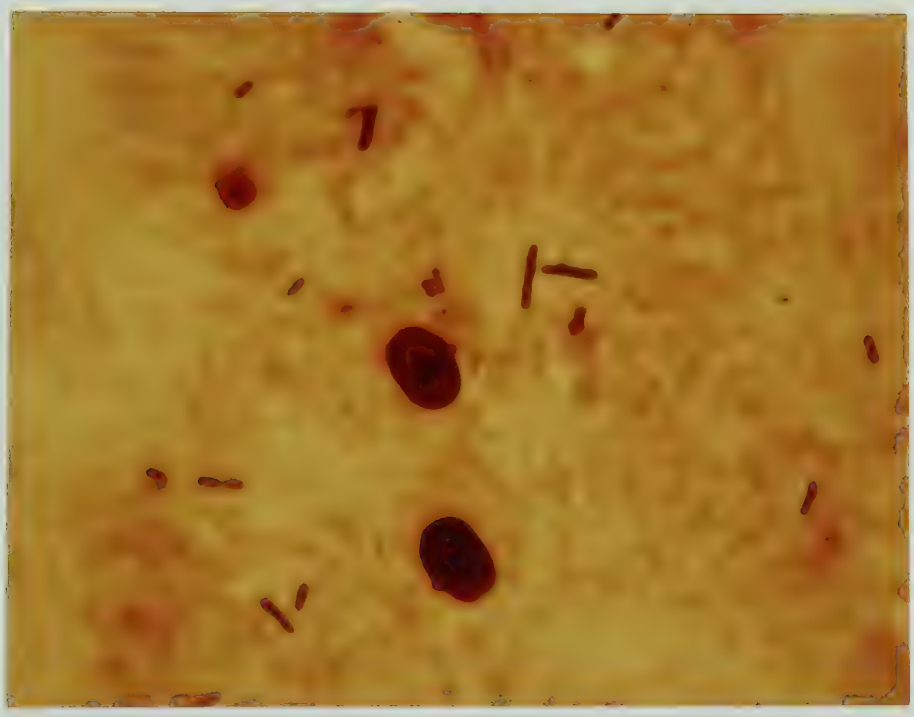
Table 3. Percent composition of caecal microflora isolated on cellobiose agar from the caeca of Clethrionomys gapperi.

Bacteria	Area	SEASON		
		Autumn	Winter	Spring
Lactobacillus	Poplar	1.3	44.6	100
	Spruce	4.2	14.8	64.6
Streptococcus	Poplar	39.2	55.4	0.0
	Spruce	91.6	83.2	34.4
Enterobacteriaceae	Poplar	59.5	0.0	0.0
	Spruce	4.2	2.0	1.0

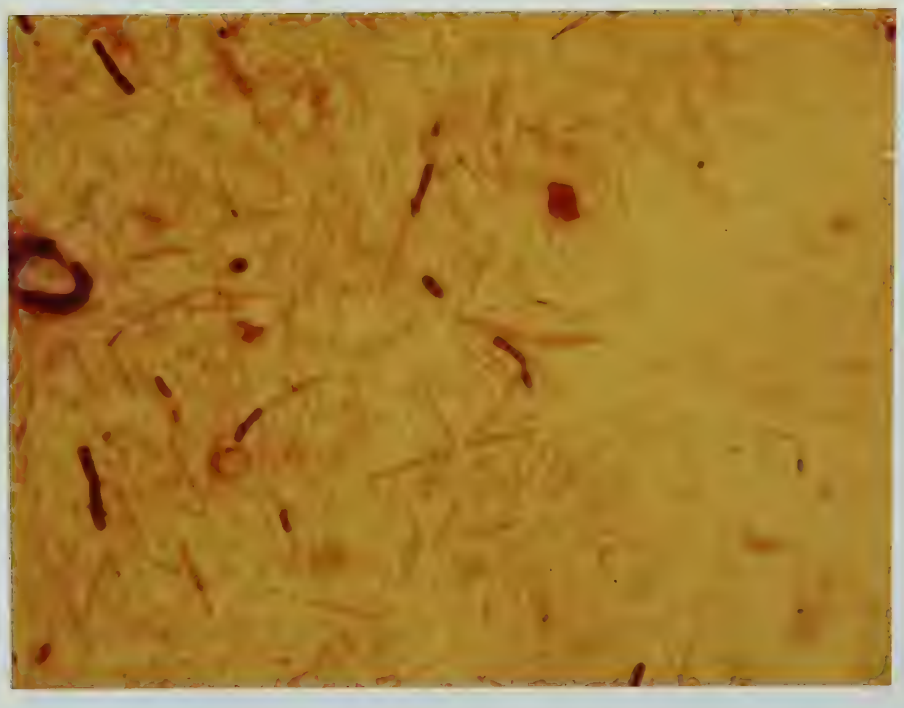
Caecal Smears

Examination of the Gram stained smears of the caecal contents showed that Gram negative, spindle-shaped rods (members of the bacteroides group) were the most numerous organisms in the caecum. Several sizes can be seen in Fig. 3. They outnumber Gram positive, non-sporing rods (lactobacilli) by a factor of 100 or more. Thus the bacteroides were present at about 10^{10} organisms per gram of caecal contents. Because their ends are pointed they belong to the genus Fusobacterium (Breed et al., 1957). A few Gram positive, sporing rods are also observable as well as very large variably Gram staining cocci. Also seen are large structures which are possibly oocysts of a sporozoan such as Eimeria (Chandler and Read, 1961).

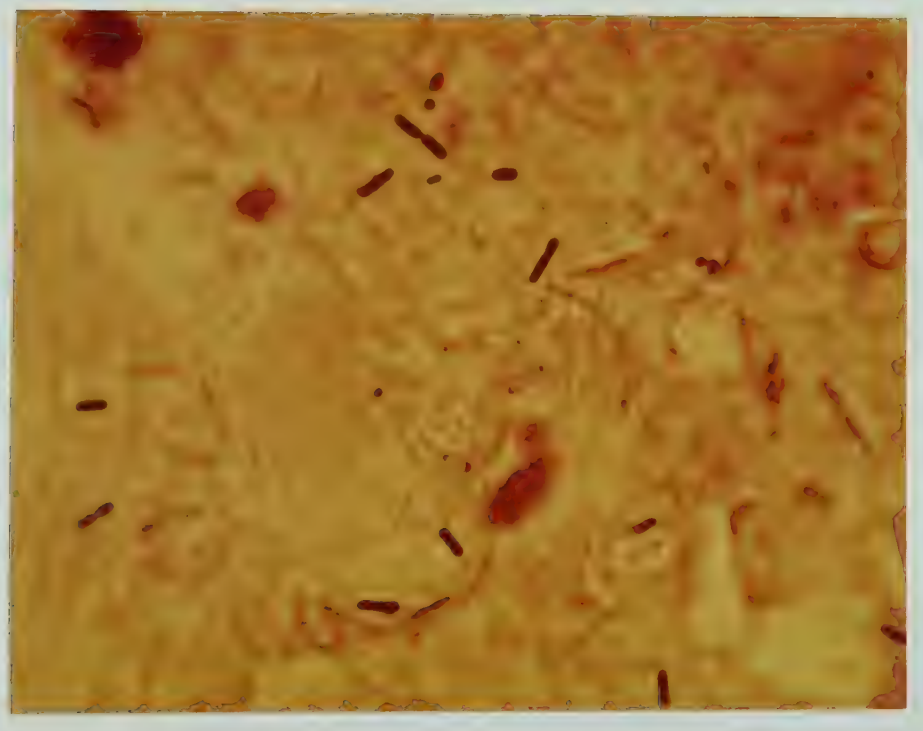
Fig. 3. Gram stained smears of the caecal contents of *Clethrionomys gapperi* (1200 X).



3a. Note the abundance of Gram negative rods of Bacteroides relative to the number of Gram positive rods of Lactobacillus.



3b. Note the various sizes of Gram negative rods. The Gram positive rod with an unstained portion is probably a Clostridium and the clear area a spore which will not take this stain.



3c. Note the abundance of Gram negative bacteria. The structures with dark tips are unidentified bipolar staining organisms.

DISCUSSION

Results of this study indicate that significant change occurs in the caecum from one season to the next. The weight and length of the caecum increased from autumn to winter and decreased after spring. The ratios varied by decreasing in value from autumn to winter and increasing in the spring. The ratios were set with the larger value in the numerator to avoid the difficulty of handling numbers less than one.

These changes were probably due to change in the fiber content of the animal's food. The chemical content of native forage in Saskatchewan and Alberta was studied by Clark and Tisdale (1945). They found that the crude fiber content was lowest in the early leaf stage, increased with growth over the summer and was a maximum after winter. Similar findings were reported for prairie forage by Smoliak and Bezeau (1967) and for alpine tundra plants by Johnston *et al.* (1958). During this same period the protein, ether extract and water soluble carbohydrates decreased (Clark and Tisdale, 1945; Bowden *et al.*, 1968). *Clethrionomys gapperi* would not only have more fiber in its food from autumn to spring but would have to eat more food to gain adequate nourishment. This may explain the changes in the caecum from one season to the next. The only other known report of seasonal change in caecum size was for California quail,

Lophortyx californicus, whose small intestine and caeca were longer in winter when their diet was of low quality and high bulk (Lewin, 1963).

Caecal length is probably a better indication of increased food bulk than caecal weight. The amount of food in the caecum will vary throughout the day as it is evacuated and refilled. But the whole structure will be stretched to a size that will accommodate the usual daily load. The ratio of body length to caecal length provides a useful index for comparing animals on different diets from different age groups, habitats and seasons. The length of the tail is excluded from this value, for it is an appendage and can independently vary in length. The caecum should grow in proportion to the body's growth and vary only as the food bulk varies. A ratio by weight is not as good an indicator or as sensitive an indicator of food bulk differences. This is so because the body can vary in weight for many reasons and always includes the weight of the caecum. Caecal weight alone is a good indicator of the bulk of different diets except that it is not readily possible to compare animals of different age groups or weights.

There was a significant difference in the caecal length of animals from different habitats in winter. Unfortunately, the measurement was not made on the first animals captured; those from the poplar habitat in the autumn. The relative

lengths are significantly different between seasons. During winter the caecum of many animals in the spruce habitat was longer than the body. I expect that this diet included a great deal of fiber. In this habitat the fiber content of the food must vary a great deal between seasons to give relative-length values of 1.14, 1.00 and 1.32. Possibly animals in the spruce habitat had more fiber in their diet than animals in the poplar habitat during winter.

Caecal weights were higher in winter than in autumn and spring. This is also an indication of a greater volume of food in winter than during the other seasons. The relative caecal weight also changed greatly between seasons except from autumn to winter in the spruce habitat. The change in absolute caecal weight from winter to spring was not as great as the relative weight change. I expect the fiber content of the diet decreased when fresh vegetation appeared in the spring. During this interval the animals showed a significant change in body weight. Sealander (1966) has shown a yearly cycle in body weight for Cl. rutilus in Alaska which was directly correlated with the ambient temperature of the macroenvironment. This has also been shown by Fuller (1969) for animals near Heart L., N.W.T.

Microbiology

The normal microflora includes those organisms that are so common in an environment that they become established

in practically all of its members (Dubos, 1965) and includes the autochthonous bacteria. But the final composition is determined by the species of animal and its diet. Smith (1965a) found, while studying different animals, that organisms entering the mouth had a variable effect in determining the alimentary flora. The anatomy and physiology of the stomach has a great influence as does the food eaten and the practise of coprophagy (Lev et al., 1966). Protein in a diet favours growth of enterobacteria (Porter and Rettger, 1940) and clostridia (Smith, 1965a). Dubos (1965) reported that coliform bacilli, streptococci, staphylococci, molds and yeasts are less numerous in each section of the intestine of pigs fed lactose as compared to those receiving starch. In contrast, the number of lactobacilli and other anaerobes is high on a diet containing lactose. Smith (1965a) also reported that cereal starches favour growth of yeasts, as seen in guinea-pigs. Lactobacilli are also favoured by an acid pH in the stomach (Dubos and Schaedler, 1962) and by coprophagy (Smith, 1965a) but the latter is not necessary (Lev et al., 1966).

Table 2 gives the normal flora, to the extent that it was studied, for Clethrionomys gapperi. Other media and techniques may show the presence of other bacteria. The genera of the family Enterobacteriaceae are usually the

least abundant as shown by growth on MacConkey Agar. The presence of a yeast, Rhodotorula glutinis, is shown by growth on Phytone Yeast Extract Agar. It is more abundant than the enterobacteria but occurs in low numbers also. In increasing order of abundance we find aerobic species of Staphylococcus and Streptococcus isolated on Sheep Blood Agar; anaerobic genera of Streptococcus and Lactobacillus, isolated by anaerobic incubation of Sheep Blood Agar; Lactobacillus on Rogosa Agar and Fusobacteria seen in stained smears of caecal contents. It is evident that anaerobic bacteria are more abundant than aerobic bacteria.

The growth on MacConkey Agar was identified as species of the genera Aerobacter and Citrobacter. They were the least numerous of the organisms isolated. The source available to the host would be feces of other animals and soil (Holding, 1960) and both sources could contaminate plants. Because growth of enterobacteria is favoured by a protein diet, their low numbers may serve as an indication of little protein in the host's diet. Higher numbers present in the spring might be related to the higher protein content of young vegetation. It could also be an indication of greater contamination carried in spring run-off water.

Only one species of yeast, Rhodotorula glutinis, was isolated from the caecum. It was most likely derived from the skin of small fruit. Because their numbers are low

we might speculate that starch, or seeds, make up a small portion of the diet of Clethrionomys. Cells of yeast provide a source of B vitamins and stimulate the growth of certain bacteria such as Lactobacillus (Difco Manual, 1953, p. 270).

Aerobic incubation of Sheep Blood Agar supported the growth of species of Staphylococcus and Streptococcus. Staphylococcus epidermidis is a common inhabitant of human skin (Sarkany and Gaylord, 1968) and probably of other mammals. Contact with the mother and other animals and their feces is the likely source of this organism for the host. Species of Streptococcus are part of the autochthonous intestinal flora of mammals. They would be found in all animal feces, in water, soil and on plants in contact with feces. Streptococci vary from aerobic to microaerophilic. In vitro their growth is favoured by carbohydrates from which they produce acid (Skerman, 1959).

Species of Streptococcus and Lactobacillus were isolated on anaerobically incubated Sheep Blood Agar and Lactobacillus on anaerobically incubated Rogosa Agar. The latter is selective in being strongly acidic. Both genera are part of the autochthonous flora and require an environment with a low oxygen tension. Growth of lactobacilli is favoured by carbohydrates and yeast extract (Skerman, 1959) and produces a final pH below 6.0 in vitro with the production of lactic acid alone or with acetic acid and alcohol.

The acid produces an environment in which they can survive, but one that is inhibitory to many other genera (Donaldson, 1964). However, it is probably the use of oxygen by aerobic bacteria that maintains the anaerobic environment needed by all autochthonous bacteria.

Anaerobic Cellobiose Agar gave the highest counts. Therefore it must have provided the most suitable environment for growth of caecal bacteria. Sheep Blood Agar provides many nutrients needed for growth by fastidious bacteria yet its anaerobic incubation supported growth at a much lower dilution. Even the Rogosa Agar was less suitable for lactobacilli than the ACA. One possible explanation for this is the more absolute anaerobic environment provided by ACA in roll-tubes.

The most numerous organisms in the caecum, members of the bacteroides group, were never isolated on this medium but were evident in the Gram stained smears. Because their ends were pointed they are likely members of the genus Fusobacterium (Breed et al., 1957). Members of the bacteroides group, which includes the genera Bacteroides, Fusobacterium, Sphaerophorus and Dialister (Skerman, 1959) are reported to be the predominant bacteria in the caecum, large intestine and feces of most mammals (Dubos et al., 1965; Hagen et al., 1965; Schaedler et al., 1965; Smith, 1965a, 1965b) including humans (Rosebury, 1962). However these organisms have been little studied in Anglo-Saxon

countries and there are no dependable and convenient cultural techniques for them (Dubos et al., 1967). Finegold et al. (1965) record many attempts to produce a selective medium for Bacteroides and other anaerobes. His own method involves the incorporation of the antibiotics neomycin, paromomycin or kanomycin alone or in blood agar with 5% sheep blood. Fredette (1956) and Quinto (1964) advocate the use of French methods for the identification of nonsporulating anaerobes. The authority in this field is Prevot (1966; translated by Fredette) whose classification of these organisms is quite different and more extensive than that of Breed et al. (1957). His method involves use of heat-sealed glass vials and cultural tests not used routinely in Anglo-Saxon countries. Because of the different classification, new tests and procedures to learn, his method was not used.

Moore (1966) proposed the use of the methods of Hungate (1950) which were used in studying anaerobic rumen microflora. Hall (1952) used the Hungate methods to study cellulolytic bacteria in laboratory rabbits. Spears and Freter (1967) also suggested this method for "Improved isolation of anaerobic bacteria from the mouse caecum..." and they used the medium of Bryant and Robinson (1963). It is the ACA medium used in this study and presented by Bryant (1963) where he reports the successful isolation

of many anaerobic bacteria including species of the genera Bacteroides and Fusobacterium. Failure to isolate these bacteria from the caecum of Clethrionomys must be due to unidentified chemical differences in the contents of the rumen and caecum. Spears and Freter (1967) did not identify any of their isolates and merely recorded higher anaerobic counts. The source of these bacteria for young animals is likely the feces of adults. Their role in the microbial ecology of the caecum is not yet known.

Members of the genera Lactobacillus and Streptococcus, isolated on ACA, were the second most numerous organisms in the caecum at different times, lactobacilli replacing the streptococci during the three seasons studied. The abundance of this organism has been reported by others as mentioned in the literature review. After studying laboratory mice which were fed a diet of constant composition Dubos et al. (1965) reported, "These three bacterial species (genera) - - - Bacteroides, Lactobacillus and Streptococcus - - - persist in approximately constant levels in their characteristic localization throughout the life span of healthy animals." Clethrionomys has the same autochthonous flora as reported for other animals. But with a changing diet through the year the relative numbers of these organisms will also change. It is not possible at this time to explain the relative number of the various species of bacteria and their seasonal and habitat variations in terms of food eaten.

A full understanding of such differences would require a chemical study of the plants eaten and of stomach and caecal contents in conjunction with a microbiological study. Intestinal bacteria live in a chemical environment. Each of the bacteria isolated is capable of utilizing many different sources of carbon (Breed *et al.*, 1957).

The non-autochthonous flora can be considered as accidental residents (Ketyi, 1964). They are potential pathogens but usually associated with "sub-clinical" infections. An example of this is the effect of endotoxin production by members of the family Enterobacteriaceae (Saltz *et al.*, 1965; Carter and Einheber, 1966; Truscott and Inniss, 1967; Markley *et al.*, 1967). This is the reason for death of guinea-pigs after penicillin treatment and sickness of most experimental animals. Farrar and Kent (1965) reported that in the first 12 hours after penicillin administration the total number of culturable organisms (predominantly Gram positive bacteria) in the caecum fell to less than 1% of the pretreatment level. Between 24 and 48 hours a ten million fold increase occurred in the number of coliform bacteria in the caecum. This resulted in bacteremia, pathological changes and eventual death. The diet of *Cl. gapperi*, as for *Cl. glareolus* includes tree bark, buds, lichens, etc. which are rich in various bacteriostatic and bactericidal substances (Kunicki-Goldfinger and Kunicka-Goldfinger, 1962c;

Oh, 1968) and may selectively change the intestinal microbial population.

Although no cellulolytic bacteria were isolated, all of the isolates possessed the enzyme cellobiase. Maybe that is the only enzyme they require to live in the caecum of Clethrionomys. Since their host is quite small it must grind its food into very small particles to pass through its oesophagus. In the host's stomach secretions of acid would degrade the cellulose and possibly leave little cellulose but much cellobiose. Kunicki-Goldfinger and Kunicka-Goldfinger (1962b, 1962c) found cellulolytic species of Clostridium in the caecum of Clethrionomys glareolus. They found their numbers increased from autumn to winter and persisted through the spring. This was unexpected since the spring diet resembled the Polish summer-autumn diet.

An increase in the number of autochthonous bacteria in an animal, while the nutrition is becoming poorer, is an advantage to the host. The increase in action of bacteria on cellobiose, etc. will increase the amount of vitamins synthesized which the host can absorb. If they are coprophagic, as are rats and lagomorphs, their diet will also gain the bacteria as a protein source. Presumably they are also producing volatile fatty acids which will be absorbed and used as an energy source. In the porcupine VFA accounted for 33% of the maintenance

energy (Johnson and McBee, 1967).

Any change in the host's environment that will change the food eaten by the host will cause a change in the intestinal environment. Bacteria live in a chemical mileu and any change in it will result in an adaptive change in the bacterial population. Brock (1966) says the change will show in several ways. Individual cells may synthesize new enzymes, a physiological adaptation; or there may be selection of mutants, a genetic adaptation. Third, a change in the environment may make conditions favourable for an organism which was initially present in small numbers because it could compete only poorly but which in the new environment is able to compete well. This has been called sociological adaptation by Wuhrmann (1964) and is probably the most important type of adaptation. Ruminants such as cattle and sheep that live in a man-made environment may suffer from a condition of bloat in the spring. This condition occurs when their food is suddenly changed, within one day, from cured hay to fresh grass. Untreated chronic bloat usually causes sudden death (see Bloat). This condition would be rare in wild ruminants in which the transition in food would be gradual as the season comes on. This condition would also be rare in non-ruminant herbivores, as a quantitative change in fibrous material would occur with a change in season and this

would cause a distention of the caecum and a quantitative change in the bacterial population. However, if a sudden change in food occurred, within one day, possibly by a late freezing rain in the spring which would cover fresh vegetation, then the host might suffer. A sudden increase in fiber could cause great distention of the caecum and possible blocking of the intestine. If the bacterial population could not respond fast enough to break down the fiber the host may suffer nutritionally. In a period of environmental stress such as cold, rain and no insulating snow, a metabolic stress by an increase in maintenance energy requirement may be the reason for many deaths in the population.

Until the present study, all work on the normal microflora of rodents has been on laboratory animals except that of Kunicki-Goldfinger and Kunicka-Goldfinger (1962a, 1962b, 1962c). Laboratory animals live in an artificial environment with artificial food that has been sterilized at least once in its preparation. Wild rodents living in a natural environment eat many kinds of animals and plants all of which contribute bacteria that will pass into their intestine. There is an opportunity for development of an intestinal microflora that could differ greatly from that reported for laboratory animals. It is noteworthy that wild and laboratory animals have a similar autochthonous microflora, or, stated in a truer perspective, that laboratory

animals still have the same symbiotic relationship after so many generations in the laboratory environment. The findings in the laboratory could not automatically be expected to exist in the wild. This study had to be carried out to determine the character of the microflora. Now that we know that the microflora structure is of the same character, we can hypothesize that it will have a similar function. By this symbiotic relationship, the host, Clethrionomys gapperi, can maintain its well-being over a time of poor food resources. It thus avoids malnutrition and can resist the stress of the physical environment, bacterial infections and some parasitic infections.

Summary

1. Clethrionomys gapperi were live-trapped during three consecutive seasons; autumn, winter and spring and in two habitats; a black spruce-feathermoss consociation and a white poplar stand.

2. The caecum weight and length were measured and compared with the animal's weight and length. The weight and length of the caecum increased and then decreased over the study period. In the Spruce habitat the values were 0.68 gram, 0.94 gram and 0.37 gram and 75.8 cm, 89.1 cm and 80.0 cm respectively. The ratio of weights (20.0, 19.9, 58.5) and lengths (1.14, 1.00, 1.32) also showed a variation from one season to the next. This was attributed to an increase in food intake and in fiber content of the food during the winter and a subsequent decrease the following spring.

3. Microbiological studies were conducted on the caecal contents using blood agar, nutrient agar, MacConkey agar, Rogosa agar and Phytone Yeast extract. The Hungate method was used for culturing with Cellobiose agar in anaerobic roll-tubes. Mastery of this technique was necessary as a much lower oxidation-reduction potential can be attained and maintained for a long time. Each medium supported the growth of one or more of Lactobacillus spp., Streptococcus spp., Staphylococcus spp., members of the Enterobacteriaceae

and the yeast Rhodotorula glutinis. Their numbers ranged from 10^2/gram for the Enterobacteriaceae to 10^8/gram for Lactobacillus spp. Anaerobic bacteria were more numerous than aerobic bacteria.

4. Gram stained smears of the caecal contents showed the most prominent organisms in the caecum to be members of the bacteroides group which were estimated to be in the range of 10^{10}/gram of caecal contents.

5. The most numerous organisms cultured were lactobacilli and streptococci. They, along with the bacteroides group, constitute the autochthonous intestinal flora. Their total number increased over the winter season. This is coincident with an increase in fiber content of the food of the host and a decrease in protein, fat and sugar content.

6. This symbiotic relationship is to the host's advantage. These bacteria enable Clethrionomys to supplement its nourishment with vitamins, volatile fatty acids and bacterial protein. They also suppress other intestinal bacteria and some parasitic infections.

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APPENDIX A

General Purpose Medium Containing Standardized Ingredients (Bryant, 1963)

Ingredients		Final Concentration
1. Energy source - glucose, cellobiose, maltose, lactic acid, etc.		0.05% - 1.0%
2. Organic nitrogen and vitamin sources (a)		
	Trypticase or equivalent	0.1 - 2.0%
	Yeast extract	0.1 - 0.2%
	Hemin	0.1 mg %
	Menadione	0.1 mg %
3. Salt solution -	$(NH_4)_2SO_4$, KH_2PO_4, NaCl	0.09 %
	$CaCl_2$, $MgCl_2.6H_2O$	0.002 %
	$MnCl_2.4H_2O$	0.001 %
	$CoCl_2.6H_2O$	0.0001 %
	$FeSO_4.7H_2O$	0.01 %
The latter three salts can be deleted from most media.		
4. Resazurin		0.0001 - 0.0002%
5. Acetic acid (glacial), Isobutyric, isovaleric, DL - alpha - methyl-butyric, n-valeric acid, "pure", each		0.17 % v/v 0.01 % v/v
6. Add water, adjust to pH 6.5 - 6.7, make to final volume minus volumes of carbonate and reducing agent solutions.		
7. Autoclave at 15 lb. for 15-20 min. and cool under O_2-free conditions.		

Appendix A (Cont'd.)

Ingredients	Final Concentration
8. Add sterile, O_2-free solution Na_2CO_3 (b)	0.06 %
9. Add sterile CO_2-free solution of one or more reducing agents.	
Cysteine. $HCl.H_2O$	0.05 %
$Na_2S.9H_2$	0.025%
$Na_2S_2O_3$	0.003%

a. Organic nitrogen sources are often not required, and low levels of trypticase will satisfy the nitrogen and vitamin requirements of many species.

b. The level of $NaHCO_3$ or Na_2CO_3 added depends on the gaseous phase of the medium and the pH desired.

APPENDIX B

Measurement	Habitat	Values	SEASON Autumn	Winter	Spring
Body Weight	Poplar	n	6	15	5
		Σ	101.4	225.5	103.25
		$\overline{X}$	16.9	15.03	20.65
		ΣX^2	1721.3	3412.32	2137.08
		$(\Sigma X)^2/n$	1713.66	3390.02	2132.11
		Σ_x	7.67	22.30	4.97
	Spruce	n	6	14	5
		Σ	78.90	194.9	91.95
		$\overline{X}$	13.15	13.92	18.39
		ΣX^2	1049.59	2773.87	1705.69
		$(\Sigma X)^2/n$	1037.53	2713.28	1690.96
		Σx	12.06	60.58	14.73
Caecal Weight	Poplar	n	4	14	3
		Σ	2.1	13.1	1.6
		$\overline{X}$	0.52	0.94	0.53
		ΣX^2	1.1350	13.83	0.90
		$(\Sigma X)^2/n$	1.1025	12.2578	0.8533
		Σx	0.0325	1.5722	0.0467
	Spruce	n	6	14	5
		Σ	4.05	13.1	1.85
		$\overline{X}$	0.68	0.94	0.37
		ΣX^2	2.8613	14.7300	0.7775
		$(\Sigma X)^2/n$	2.7337	12.2578	0.6845
		Σx	0.1276	2.4722	0.0930
Body Weight / Caecal Weight	Poplar	n	4	14	3
		Σ	132.89	273.7	123.18
		$\overline{X}$	33.22	19.55	41.06
		ΣX^2	4620.99	7223.74	5430.93
		$(\Sigma X)^2/n$	4414.94	5350.8	5057.77
		Σx	206.05	1872.94	373.16
	Spruce	n	6	14	5
		Σ	119.92	278.11	292.37
		$\overline{X}$	19.98	19.86	58.47
		ΣX^2	2434.84	8697.01	20,611.87
		$(\Sigma X)^2/n$	2396.8	5524.65	17,096.04
		Σx	38.04	3172.36	3,515.83

APPENDIX B (Cont'd.)

Measurement	Habitat	Values	SEASON Autumn	Winter	Spring
Caecal Length	Poplar	n	--	13	3
		Σ		1041.0	250.
		$\overline{X}$		80.08	83.3
		ΣX^2		84,125.	20,900.
		$(\Sigma X)^2/n$		83,360.08	20,833.3
		Σx		0765.0	67.0
	Spruce	n	6	14	5
		Σ	455	1248.	400.
		$\overline{X}$	75.83	89.14	80.
		ΣX^2	34,625.	112,726.	32,350.
		$(\Sigma X)^2/n$	34,504.	111,250.	32,000.
		Σx	121.0	1,476.	350.
Animal Length	Poplar	n	4	15	4
		Σ	367.1	1360.	409.
		$\overline{X}$	91.77	90.66	102.25
		ΣX^2	33,745.	123,626.	42,027.
		$(\Sigma X)^2/n$	33,690.	123,306.6	41,820.25
		Σx	55.4	319.3	207.
	Spruce	n	6	14	5
		Σ	518.	1237.0	521
		$\overline{X}$	86.3	88.357	104.2
		ΣX^2	44,728.	109,619.	54,397
		$(\Sigma X)^2/n$	44,720.6	109,297.79	54,288.2
		Σx	7.4	321.21	109.
Body Length / Caecal Length	Poplar	n	--	13	3
		Σ		14.91	3.83
		$\overline{X}$		1.15	1.28
		ΣX^2		17.4299	4.921
		$(\Sigma X)^2/n$		17.1006	4.889
		Σx		0.3293	0.032
	Spruce	n	6	14	5
		Σ	6.86	14.05	6.60
		$\overline{X}$	1.14	1.00	1.32
		ΣX^2	7.8748	14.1503	8.862
		$(\Sigma X)^2/n$	7.8432	14.1001	8.712
		Σx	0.0316	0.0502	0.150